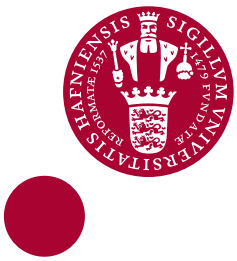

Rice leaf hydrophobicity and gas films are conferred by a wax synthesis gene (LGF1) and contribute to flood tolerance

Kurokawa, Yusuke; Nagai, Keisuke; Hung, Phung Danh; Shimazaki, Kousuke; Qu, Huangqi; Mori, Yoshinao; Toda, Yusuke; Kuroha, Takeshi; Hayashi, Nagao; Aiga, Saori; Itoh, Jun-ichi; Yoshimura, Atsushi; Sasaki-Sekimoto, Yuko; Ohta, Hiroyuki; Shimojima, Mie; Malik, Al Imran; Pedersen, Ole; Colmer, Timothy David; Ashikari, Motoyuki

Published in:
New Phytologist

DOI:
10.1111/nph.15070

Publication date:
2018

Document version
Publisher's PDF, also known as Version of record

New Phytologist

Research

Rice leaf hydrophobicity and gas films are conferred by a wax synthesis gene (*LGF1*) and contribute to flood tolerance

Yusuke Kurokawa[1], Keisuke Nagai[2], Phung Danh Hung[1,3], Kousuke Shimazaki[4], Huangqi Qu[1], Yoshinao Mori[1], Yusuke Toda[5], Takeshi Kuroha[6], Nagao Hayashi[7], Saori Aiga[8], Jun-ichi Itoh[8], Atsushi Yoshimura[9], Yuko Sasaki-Sekimoto[10,11], Hiroyuki Ohta[10,11,12], Mie Shimojima[10], Al Imran Malik[13], Ole Pedersen[14,15], Timothy David Colmer[15] and Motoyuki Ashikari[2]

[1]Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa, Nagoya, Aichi 464-8602, Japan; [2]Bioscience and Biotechnology Center, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8602, Japan; [3]Crops Research and Development Institute, Vietnam National University of Agriculture, Trau Quy, Gia Lam, Ha Noi, Vietnam; [4]Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori, Yokohama, Kanagawa 226-8503, Japan; [5]Graduate School of Science, Nagoya University, Furo-cho, Chikusa, Nagoya, Aichi 464-8602, Japan; [6]Graduate School of Life Science, Tohoku University, 6-3 Aoba, Aramaki, Aoba, Sendai, Miyagi 980-8578, Japan; [7]Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan; [8]Graduate School of Agricultural and Life Science, The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan; [9]Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi, Fukuoka 812-8581, Japan; [10]School of Life Science and Technology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori, Yokohama, Kanagawa 226-8503, Japan; [11]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Japan; [12]Earth-Life Science Institute, Tokyo Institute of Technology, 4259 Nagatsuta, Midori, Yokohama, Kanagawa 226-8503, Japan; [13]Centre for Plant Genetics and Breeding, UWA School of Agriculture and Environment, Faculty of Science, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia; [14]Department of Biology, University of Copenhagen, Universitetsparken 4, 3rd floor, Copenhagen 2100, Denmark; [15]UWA School of Agriculture and Environment, Faculty of Science, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia

Authors for correspondence:
Ole Pedersen
Tel: +45 23747641
Email: opedersen@bio.ku.dk

Timothy D. Colmer
Tel: +61 864881993
Email: timothy.colmer@uwa.edu.au

Motoyuki Ashikari
Tel: +81 527895202
Email: ashi@agri.nagoya-u.ac.jp

Received: *22 November 2017*
Accepted: *14 January 2018*

New Phytologist (2018)
doi: 10.1111/nph.15070

Key words: air film, epicuticular waxes, *Oryza sativa*, primary alcohol, submergence tolerance, superhydrophobic, underwater photosynthesis, wax biosynthesis.

Summary

- Floods impede gas (O_2 and CO_2) exchange between plants and the environment. A mechanism to enhance plant gas exchange under water comprises gas films on hydrophobic leaves, but the genetic regulation of this mechanism is unknown.
- We used a rice mutant (*dripping wet leaf 7*, *drp7*) which does not retain gas films on leaves, and its wild-type (Kinmaze), in gene discovery for this trait. Gene complementation was tested in transgenic lines. Functional properties of leaves as related to gas film retention and underwater photosynthesis were evaluated.
- *Leaf Gas Film 1* (*LGF1*) was identified as the gene determining leaf gas films. *LGF1* regulates C30 primary alcohol synthesis, which is necessary for abundant epicuticular wax platelets, leaf hydrophobicity and gas films on submerged leaves. This trait enhanced underwater photosynthesis 8.2-fold and contributes to submergence tolerance. Gene function was verified by a complementation test of *LGF1* expressed in the *drp7* mutant background, which restored C30 primary alcohol synthesis, wax platelet abundance, leaf hydrophobicity, gas film retention, and underwater photosynthesis.
- The discovery of *LGF1* provides an opportunity to better understand variation amongst rice genotypes for gas film retention ability and to target various alleles in breeding for improved submergence tolerance for yield stability in flood-prone areas.

Introduction

Water limits global agricultural production; too little or too much destroys crops. Floods alone can cause yield losses worth $US 7.8 billion every year (FAO, 2015). Even rice (*Oryza sativa*), a wetland crop which grows in shallow standing water and feeds a large proportion of the world population, frequently suffers damage or loss in flood-prone regions (Ismail *et al.*, 2013). Inundation by water impedes shoot gas exchange, which slows CO_2 entry and restricts photosynthesis during the day and slows O_2 uptake, resulting in tissue hypoxia during the night (Pedersen *et al.*, 2009; Colmer *et al.*, 2014). A mechanism to enhance tissue gas exchange with water, evident for both aquatic invertebrate animals and plants, comprises hydrophobic body surfaces that

retain a thin layer of gas; for plants, the term 'leaf gas films' has been used to describe this trait (Pedersen *et al.*, 2009; Pedersen & Colmer, 2012). The surface of rice leaves is hydrophobic and the leaves initially retain gas films when submerged (Supporting Information Fig. S1), but the gas layer can diminish with time under water and result in reduced photosynthesis (Winkel *et al.*, 2014) and a lower O_2 status (Winkel *et al.*, 2013). Research on the physiological roles of leaf gas films has previously relied on experimental removal of surface hydrophobicity using a dilute detergent so that gas films were not formed during submergence (Raskin & Kende, 1983; Colmer & Pedersen, 2008; Pedersen *et al.*, 2009). However, a molecular genetic approach using a rice mutant with diminished capacity to retain leaf gas films will enable elucidation of the genetic regulation of this leaf trait and improve knowledge of the physiological function in plant submergence tolerance.

Leaf hydrophobicity has been studied in detail for some species (e.g. sacred lotus; Barthlott & Neinhuis, 1997) and it occurs as a result of various macro-, micro- and nanostructures on the surface – plicate leaves, papillae and epicuticular waxes, respectively (Marmur, 2003; Koch & Barthlott, 2009) – although the epicuticular wax platelets are considered to be of particular importance (Koch & Barthlott, 2009; Herzog *et al.*, 2017). Synthesis of epicuticular waxes involves fatty acyl-CoA elongation, the products of which are then catalysed to primary alcohols by fatty acyl reductases, a process that in Arabidopsis is controlled by members of the *CER* gene family (Samuels *et al.*, 2008). *ECERIFERUM4* (*CER4*) encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in epicuticular wax production in Arabidopsis, specifically in conversion of C26 and C28 very long-chain fatty acyl-CoA to the respective (same C number) primary alcohols (Rowland *et al.*, 2006). In rice, *OsHSD1* encodes a hydroxysteroid dehydrogenase (HSD) that influences leaf wax composition, which was suggested to occur via interactive effects of OsHSD1 on expression of *CER* genes (Zhang *et al.*, 2016). Leaf surface hydrophobicity was lost in an *oshsd1* mutant (Zhang *et al.*, 2016), but the influence on leaf gas films, underwater photosynthesis and submergence tolerance was not evaluated. The importance of leaf hydrophobicity and gas films for underwater photosynthesis and submergence tolerance of rice (Pedersen *et al.*, 2009; Winkel *et al.*, 2013; Colmer *et al.*, 2014) and some other species (Colmer & Pedersen, 2008; Colmer *et al.*, 2011) prompted the present study, which aimed to identify a gene conferring leaf gas film retention in rice and its phenotypic influence.

The present study used a rice mutant (*dripping wet leaf 7*, *drp7*) with leaves that were significantly less hydrophobic than the wild-type (Satoh *et al.*, 1983), evident as water adhesion rather than water shedding during rainfall. We tested the hypothesis that upon submergence the *drp7* mutant does not retain leaf gas films and elucidated the greatly reduced gas film retention phenotype of the *drp7* mutant, followed by gene mapping and a gene complementation test. Discovery of the gene determining leaf gas film retention, *Leaf Gas Film 1* (*LGF1*), demonstrated that *LGF1* determines leaf wax composition, epicuticular wax platelet abundance, surface hydrophobicity, and thus gas film retention and underwater photosynthesis for leaves of rice.

Materials and Methods

Plant material

Rice (*Oryza sativa* L.) cv Kinmaze and the *drp7* mutant (Satoh *et al.*, 1983) from a *N*-methyl-*N*-nitrourea mutagenized population were provided by the Laboratory of Plant Breeding, Kyushu University. Other rice materials used to generate a mapping population and to make transgenic plants for a gene complementation test are described in the relevant sections later in the paper. Plants were grown in pots of flooded rice paddy soil in trays of deionized water either in a glasshouse (June-to-August, Nagoya, Japan) for the mapping population and the transgenic plants, or in a controlled-environment room (30 : 25°C, light : dark periods; 16 h light period of 100 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR)) for the characterization of the leaves of Kinmaze and the *drp7* mutant.

Leaf hydrophobicity and gas film thickness

Hydrophobicity of the leaf cuticle was assessed using the contact angle of small droplets of water, and contact angles > 90° indicate a hydrophobic surface and those > 150° indicate superhydrophobicity (Koch & Barthlott, 2009). Hydrophobicity of leaf blade segments of Kinmaze, *drp7*, *pUb*(VC) and *pUb*::*LGF1* was quantified by measuring the contact angle of a 1 μl water droplet on the adaxial side with a wettability measurement system (LSE-ME3; Nick, Kanayamacho12-1, Kawaguchishi, Japan). The contact angle of each sample was calculated by averaging the values of three measurements for 5 s. Samples were from the youngest fully expanded leaf of rice at the seventh leaf stage.

Gas film thickness on submerged leaf blade segments was measured using the buoyancy method (Raskin, 1983; Colmer & Pedersen, 2008). The buoyancy of leaf segments (*c.* 5 cm in length) taken around one-third of the distance from the tip of the youngest fully expanded leaf was measured in deionized water with the sample mounted on a hook underneath a four-digit balance with measurements before and after brushing both surfaces of the leaf segment with 0.1% (v/v) Triton X-100 to remove any gas films. Leaf gas film thickness was calculated as: gas volume (m^3)/[2 × projected area (m^2)] (see Colmer & Pedersen (2008) for details).

Leaf net photosynthesis: under water and in air

Underwater net photosynthesis was assessed by measuring O_2 evolution in a closed system according to the method of Pedersen *et al.* (2013). In brief, leaf blade segments (*c.* 2.5 cm in length) taken around one-third of the distance from the tip of the youngest fully expanded leaf were inserted into glass bottles which contained 25 ml of medium, and two glass beads were added to ensure mixing as the bottles rotated inside the illuminated water bath at 30°C; one leaf segment was placed in each

bottle. The PAR inside the glass bottles was 1000 μmol m^{-2} s^{-1} (measured using a spherical sensor; 4p US-SQS/L; Walz, Effeltrich, Germany). The incubation medium was based on the general purpose culture medium described by Smart & Barko (1985) and contained (in mmol m^{-3}): Ca^{2+}, 0.62; Mg^{2+}, 0.28; Cl^-, 1.24; SO_4^{2-}, 0.28. Various amounts of $KHCO_3$ were added to the incubation medium, and 0.1 M HCl was used to adjust the pH (6.3–8.7), thus converting HCO_3^- into free CO_2 to achieve a range of dissolved CO_2 (0.5–2500 mmol m^{-3}), whilst keeping HCO_3^- constant at 2.0 mol m^{-3}. The dissolved O_2 concentration in the incubation medium was set at *c.* 50% of air equilibrium applied to prevent an increase in O_2 above air equilibrium levels during measurements that might have led to photorespiration and thus decreased net photosynthesis (Colmer & Pedersen, 2008). Following incubations of known duration, the dissolved O_2 concentration in each bottle was measured using a calibrated O_2 mini-electrode (OX-500; Unisense A/S, Aarhus, Denmark) connected to a pico-amperemeter (PA2000; Unisense A/S). Dissolved O_2 concentrations in bottles prepared and incubated in the same way, but without leaf segments, served as blanks. The projected area of each leaf segment was measured using digital photography followed by analysis with ImageJ software (Schneider *et al.*, 2012).

Rates of net photosynthesis in air were measured about one-third of the distance from the tip of the youngest fully expanded leaf blade on the main stem of rice. A Li-Cor 6400XT (Li-Cor, Lincoln, NE, USA) photosynthesis system was used with an external light source of 1000 μmol m^{-2} s^{-1} PAR, block temperature of 28°C, flow rate of 500 μmol s^{-1}, CO_2 of 800 μmol mol^{-1} (twice atmospheric concentrations to overcome any stomatal limitations on CO_2 entry, as the aim was to evaluate the capacity of the photosynthetic machinery in air for benchmarking against maximal rates when under water), and relative humidity (RH) of 80%. In some experiments net photosynthesis in air was measured with ambient CO_2 at 400 μmol mol^{-1} and 46% RH (Kinmaze and *drp7* mutant) or 80% RH (complementation lines and vector controls), with flow rate, block temperature and PAR as before.

Scanning electron microscopy of leaf surfaces

Surface structures (papillae and epicuticular wax platelets) on the adaxial surface of leaf blades were visualized using a scanning electron microscope. Samples were taken at around one-third of the distance from the tip of the youngest fully expanded leaf blade of rice at two growth stages (see relevant figure captions) and were gold-coated by Smart Coater (Jeol, Musashino, Akishimashi, Tokyo, Japan) for 2 min and viewed with a scanning electron microscope (JCM-6000; Jeol), operating at an accelerating voltage of 15 kV under high vacuum mode.

Identification of *LGF1*: gene mapping, transcript abundance, and complementation test

In order to map the *LGF1* gene, F_2 plants of crosses between the *drp7* mutant (*japonica*) and Kasalath (*indica*) were used. A total of 5300 individual plants of the F_2 population were used in positional cloning of *LGF1*. Genomic DNA was extracted from each plant of the F_2 population using the modified TPS method (Thomson & Henry, 1995; Nagai *et al.*, 2012). The purified DNA samples were then genotyped using molecular markers. PCR-based markers, including simple sequence repeat markers (https://www.ricebase.org), cleaved amplified polymorphic sequence (CAPS) markers (for this study, CAPS markers were designed using dCAPS Finder 2.0 and single nucleotide polymorphisms, which were identified by comparing the genomic DNA sequences of each parent, were used for mapping.

To confirm the coding sequences of each gene in the candidate region, we designed the sequence primers (Table S1) and amplified the genomic regions using genomic DNA from the *drp7* mutant. Amplified DNA fragments were electrophoresed and purified using Wizard SV Gel and PCR Clean-up System Kit (Promega). Sequencing of DNA fragments was performed using an ABI3730xl capillary DNA sequencer (Applied Biosystems, Waltham, MA, USA). Sequences of candidate genes were compared with annotated sequences on Rap-db (http://rapdb.dna.affrc.go.jp) and Tigr (http://rice.plantbiology.msu.edu) using Atsq software (Genetyx, Tokyo, Japan).

Total RNA was isolated from roots, youngest fully expanded leaf sheath and leaf blade and expanding leaf blade of Kinmaze and the *drp7* mutant grown in a glasshouse for 2 months, using the RNeasy Plant Mini Kit (Qiagen). First-strand cDNA was generated using Omniscript RT kit (Qiagen). Real-time quantitative PCR analysis was carried out using StepOnePlus (Thermo Fisher, Waltham, MA, USA). The cDNAs of interest were specifically amplified with the following primers: LGF1-RT-F (5′-TCAGCAAGAAGATCCTCGAG) and LGF1-RT-R (5′-CATGGCTGCGGATGGTCTTG). As a control for quantitative reverse transcription polymerase chain reaction, Ubiquitin was amplified with the primers Ubi-F (5′-AATTCCAATCCTTTCTTGCCTC) and Ubi-R (5′-TGTCAATCGTATCGGAGAAC). Each 20 μl reaction mixture contained 1 × SYBR Fast qPCR Mix, 0.4 μM gene-specific primers and 1 × ROX reference dye. Reactions were performed according to the manufacturer's instructions. The relative mRNA expression levels were normalized against Ubiquitin gene expression levels.

The full-length coding sequence of *LGF1* (*OsHSD1* (LOC_Os11g30560)) was amplified using cDNA. The primers were designed on the basis of the information on Tigr (http://rice.plantbiology.msu.edu). The coding sequence was ligated into pCAMBIA1380 containing rice Ubiquitin promoter using a DNA ligation kit (Takara, Shimogyo-ku, Kyoto, Japan), following the manufacturer's protocol. The construction was transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation (Hood *et al.*, 1986). Generation of transgenic rice plants used *Agrobacterium*-mediated transformation of rice calli according to the method described by Hiei *et al.* (1994). The transformed plants (T_0 plants) were used in experiments.

Leaf cuticular wax composition

Total cuticular wax mixtures were extracted by immersing leaf blades in chloroform for 60 s at 60°C (Zhou *et al.*, 2013). The

solution was completely evaporated under a stream of N_2 gas, and the residue was redissolved in chloroform. Wax identification and quantification were performed using published methods with minor modifications (Greer *et al.*, 2007). In thin-layer chromatography analyses, the total wax was separated on silica gel with hexane/diethyl ether/acetate (90 : 7.5 : 1, v/v/v) and each wax compound was scraped off from the silica gel after being visualized by staining with primuline and UV light. n-Tetracosane (C24 alkane) was added to the silica gel as an internal standard, and extracted with chloroform. The solution was evaporated under a stream of N_2 gas, and the residues were incubated with N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane and pyridine for 1 h at 80°C. BSTFA and pyridine were then removed under a stream of N_2 gas. The samples were dissolved in hexane and analysed for wax component identification by GC-MS (GCMSTQ-8030; Shimadzu, Nakagyouku, Kyoto, Japan), equipped with an Rxi-5HT column (length, 30 m; internal diameter, 0.25 mm; film, 0.25 μm; Restek, Benner Circle, Bellefonte, PA, USA), and for wax quantification by GC-flame ionization detection (GC-FID) (GC2014; Shimadzu), equipped with a DB-5 column (length, 30 m; internal diameter, 0.25 mm; film, 0.25 μm; Agilent, Santa Clara, CA, USA). For the GC-MS and GC-FID, the carrier gas was He, the injection port was at 320°C, and the column temperature was as follows: 50°C for 2 min, increased to 200°C at a rate of 40°C min^{-1} and held for 2 min, after which it was raised to 320°C at a rate of 3°C min^{-1} and held for 40 min. Wax quantities were determined by comparing FID peak areas against internal standards. The data were expressed per unit surface area extracted for each sample.

Sequence data

The sequence data of *LGF1* and *lgf1* have been deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession numbers LC363889 and LC363890, respectively.

Data analyses

Graphpad Prism 7.0 (La Jolla, CA, USA) was used for statistical analyses and graphing of data. Figure and table captions provide details on the various tests used and significance levels.

Results

Leaf gas films are soon lost from the *drp7* mutant when submerged and underwater photosynthesis declines

We investigated leaf gas film retention upon submergence, and leaf surface hydrophobicity for Kinmaze and the *drp7* mutant. Both genotypes possessed leaf gas films when first submerged, but the gas films on the *drp7* mutant did not persist beyond 1 d of submergence (Fig. 1a,b). Loss of the gas films was associated with a loss of leaf surface hydrophobicity (Fig. 1c–f). Water droplets were repelled from leaves of Kinmaze after 1 d of submergence, but not from the *drp7* mutant. Leaf surface hydrophobicity was quantified by contact angle measurements which showed that the *drp7* mutant had become hydrophilic after 1 d of submergence whereas Kinmaze remained hydrophobic (Fig. 1c–f). Interestingly, leaves of Kinmaze possess a higher density of epicuticular wax platelets than those of the *drp7* mutant (Figs 1g,h, S2), and these wax platelets contribute to leaf hydrophobicity (Neinhuis & Barthlott, 1997; Koch & Barthlott, 2009). Papillae density can also influence leaf hydrophobicity (Koch *et al.*, 2009), but the densities of the *drp7* mutant and Kinmaze were similar (Fig. 1g,h).

The gas film thickness on submerged leaves was maintained for Kinmaze over 4 d of submergence, whereas the *drp7* mutant did not retain gas films after 1 d of submergence (Fig. 2a). Loss of gas films caused a marked reduction in net photosynthesis of submerged leaves of the *drp7* mutant, whereas this was maintained for Kinmaze (Fig. 2b). CO_2 response curves for underwater net photosynthesis were measured for leaves after 1 d of plant submergence and showed substantially higher rates for Kinmaze than for the *drp7* mutant (Fig. 2c). Underwater net photosynthesis of Kinmaze was CO_2-saturated at 200 mmol m^{-3} in the water, whereas the *drp7* mutant required *c.* 10-fold higher external dissolved CO_2. Indeed, the resistance to CO_2 entry was 8.2-fold higher for the *drp7* mutant (gas films absent) than for Kinmaze (gas films present) with 12–200 mmol m^{-3} in the water (Fig. 2c). By contrast with the situation when submerged, capacity for net photosynthesis in air did not differ between the two genotypes (Fig. S3a). Leaf blade Chl content (soil plant analysis development, readings) and tissue thickness also did not differ between the two genotypes (Fig. S3b,c).

Identification of a gene determining leaf hydrophobicity and gas film retention

To identify the gene responsible for the *drp7* mutant phenotype of leaf gas film loss, we crossed the *drp7* mutant with Kasalath (*O. sativa Indica*), and scored the F_1 and F_2 progenies for the *dripping wet leaf* phenotype. All F_1 plants were water-repellent and for the F_2 population of 5300 individuals the segregation ratio was 3 : 1 for water-repellent, *dripping wet leaf* phenotypes, indicating a recessive mutation of a single gene, according to Mendelian inheritance. We used positional cloning with the 5300 F_2 plants to identify a candidate region of 221 kb on chromosome 11, between markers RM26764 and RM26774 (Fig. 3a). Further high-resolution mapping using dCAPS and sequencing reduced the candidate region to 154 kb (Fig. 3b), which contained 18 annotated genes in TIGR (http://rice.plantbiology.msu.edu) (Fig. 3c). Genomic DNA sequence analysis indicated a single nucleotide substitution in LOC_Os11g30560.1 (Fig. 3d) of an 'A' in Kinmaze to a 'T' in the *drp7* mutant at the 3′-splicing site in the third intron of the *OsHSD1* gene, which is a member of the short-chain dehydrogenase reductase (SDR) family (Fig. 3e). Moreover, cDNA sequence analysis indicated that this single nucleotide substitution resulted in a premature stop codon in *OsHSD1* of the *drp7* mutant, as well as a 29 bp upstream extension of the fourth exon (Fig. 3e). This premature stop codon would presumably yield a truncated protein product (Fig. S4), possibly resulting in loss of the predicted catalytic

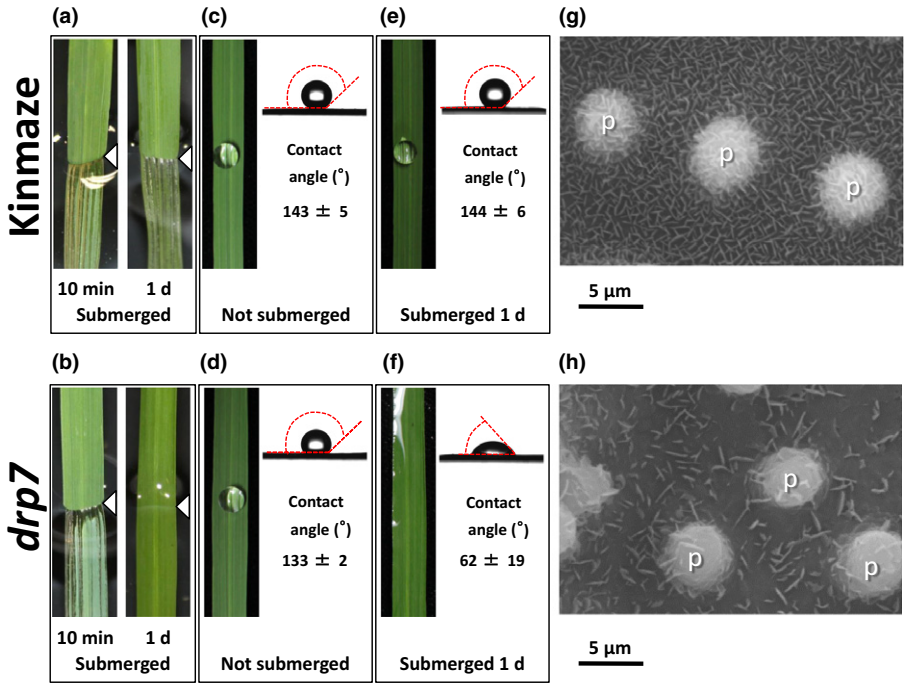

Fig. 1 Leaves of rice variety Kinmaze are hydrophobic and possess a persistent gas film when submerged, whereas for the *drp7* mutant, leaf surface hydrophobicity declines and the gas film soon collapses during submergence. (a) Gas films on the leaf blades of Kinmaze when under water (evident as a silvery sheen on the lower half of leaves dipped into water; white triangle shows water level) initially and after 1 d. (b) Gas films were initially present on the leaf blades of the *drp7* mutant when under water, but these did not persist beyond the first day of plant submergence. (c, d) The hydrophobic leaf blade surface is demonstrated by water droplets on Kinmaze and initially on the *drp7* mutant. (e, f) The leaf surface remained hydrophobic for Kinmaze when submerged, but had become hydrophilic for the *drp7* mutant during the first day under water. (c–f) Contact angle of water droplet and leaf surface were measured, as indicated by the dotted red lines. (g, h) Scanning electron micrographs of leaf blades show that Kinmaze possesses a greater density of epicuticular wax platelets than the *drp7* mutant. The numerous, small white 'flecks' on the leaf surface are the epicuticular wax platelets, whereas the larger circular-like structures (top view of a dome-like protrusion) are papillae (labelled 'p'). All measurements were on the adaxial side of leaf 7. Contact angles are of a 1 µl water droplet (side views in the figure; the top views used larger drops of water) and are the angles (relative to the horizontal) at which the water–air interface of the droplet meets the leaf surface. Data in (c)–(f) are means ± SD ($n = 4$) with each replicate being a leaf blade segment from a different plant. The decline in the mean contact angle of the *drp7* mutant was significant ($P < 0.0001$; *t*-test).

residues and thus loss of function in the mutant (i.e. loss of leaf gas films).

The present mutation differs from those reported for other rice wax mutants (Rowland *et al.*, 2006; Yu *et al.*, 2008; Islam *et al.*, 2009; Park *et al.*, 2010; Chen *et al.*, 2011; Qin *et al.*, 2011; Mao *et al.*, 2012; Zhou *et al.*, 2013; Zhu & Xiong, 2013) and is a different allele of *OsHSD1* to that recently identified by Zhang *et al.* (2016) to encode an HSD involved in wax synthesis. The importance of leaf gas films for submergence tolerance of rice (Pedersen *et al.*, 2009) and the loss of capacity for gas film retention in the *drp7* mutant (Figs 1b, 2a) prompted us to name the new allele discovered here *Leaf Gas Film 1* (*LGF1*). Consistent with its role in determining leaf cuticle wax composition, *LGF1* was preferentially expressed in the expanding leaf blade of Kinmaze (Figs 3f, S5). By contrast, the *drp7* mutant showed little expression of *LGF1*. This result suggests that the *drp7* mutant is a null mutant of *LGF1*.

Verification of the function of *LGF1/OsHSD1* by a complementation test

The function of *LGF1* was verified with a complementation test by generating transgenic lines of rice with *LGF1* cDNA from Kinmaze driven by the Ubiquitin-promotor in the *drp7* mutant (*pUb*::*LGF1*/*drp7*), as well as vector control lines in the *drp7* mutant (*pUb*(VC)/*drp7*). T_0 plants were raised in seedling trays in a glasshouse and tested for the *dripping wet leaf* phenotype as described earlier for the mapping population. T_0 plants displaying the non*dripping wet leaf* characteristic (i.e. with water-repellent leaves), as well as a random selection of T_0 vector control lines, were sampled for further measurements as described later. Gas film retention (Fig. 4a,b), leaf surface hydrophobicity (Fig. 4c–f) and density of epicuticular wax platelets (Fig. 4g,h) were all restored in the complementation lines expressing *LGF1*, but not in the vector control lines. Moreover, thickness of the gas film (Fig. 5a) and the rate of underwater net photosynthesis (Fig. 5b) were both also restored in the *LGF1* complementation lines, as compared with the vector control lines. By contrast with the enhanced photosynthesis when under water, when in air the leaves of the two lines did not differ in net photosynthesis (Fig. S6).

LGF1/OsHSD1 influences leaf wax chemistry

Leaf wax components were evaluated using GC-MS (Fig. S7) and GC-FID (Figs 6a,b and S7). The *drp7* mutant contained

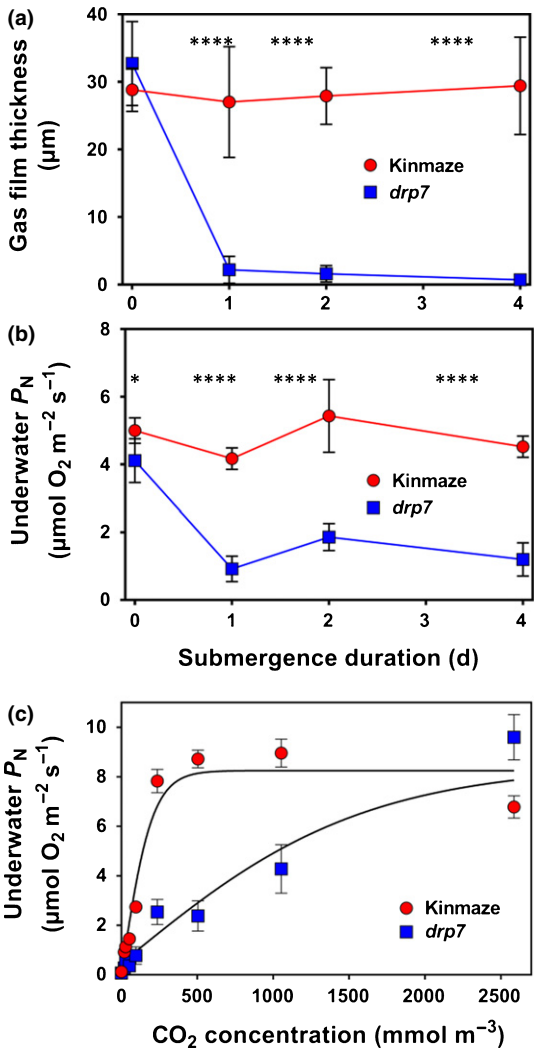

Fig. 2 Collapse of gas films on leaves of the rice mutant *drp7* results in lower rates of underwater photosynthesis than for variety Kinmaze with persistent leaf gas films. (a) Gas film thickness on leaves of Kinmaze was maintained over 4 d of plant submergence, whereas these films collapse on leaves of the *drp7* mutant. (b) Loss of leaf gas films had adverse consequences for underwater net photosynthesis (P_N) by the *drp7* mutant, whereas underwater P_N was maintained in Kinmaze. (c) Response of underwater P_N to increasing dissolved CO_2 concentrations for leaf blades of Kinmaze and the *drp7* mutant, at 1 d of submergence. The initial slope of the response curve (i.e. when CO_2 concentrations were low) shows that the rate of CO_2 entry was 8.2-fold greater in Kinmaze (with gas films) than in the *drp7* mutant (lacking gas films). A photosynthesis model (Jassby & Platt, 1976) was fitted to each dataset showing that underwater P_N is CO_2-saturated at 300 mmol CO_2 m^{-3} for Kinmaze (with gas films), whereas for the *drp7* mutant (without gas films) it only saturated near 2500 mmol CO_2 m^{-3}. The two curves converge when dissolved CO_2 was very high, because then enough CO_2 can diffuse into both leaf types for the underwater P_N. All measurements were on leaf 7. Data are means ± SD ($n = 4$ in (a); $n = 5$ (*drp7*) and 10 (Kinmaze) in (b); $n = 4$ in (c); SD bars are not visible when smaller than the symbols) with each replicate being a leaf blade segment from a different plant. Significant differences (Tukey test): *, $P < 0.05$; ****, $P < 0.0001$.

significantly less C30 primary alcohol, but more C30 aldehyde, than did Kinmaze (Fig. 6a), which was also verified by measurements of younger plants (Fig. S8). Importantly, no other wax components differed between the *drp7* mutant and Kinmaze (Figs 6a, S8). The *pUbi*::*LGF1*/*drp7* complementation lines, when compared with its vector control lines, showed more C30 primary alcohol and relatively low amounts of C30 aldehyde (Fig. 6b). These wax composition results (Fig. 6), together with the observations of epicuticular wax platelet abundances (Figs 1g, h, 4g,h), provide evidence that *LGF1* determines the balance between C30 primary alcohol and C30 aldehyde and that the C30 primary alcohol is required for formation of abundant epicuticular wax platelets, increasing leaf surface hydrophobicity and gas film retention during submergence.

Additional characterization of the *drp7* mutant

The importance of leaf hydrophobicity for paddy rice was illustrated by the death of the *drp7* mutant within 3 wk after transplantation into *c.* 15 cm of standing water in a field during the wet season in Japan, whereas Kinmaze remained green and continued to grow (Fig. S9a–c). The leaves of the *drp7* mutant could not maintain their emergence above water and eventually died, whereas leaves of Kinmaze maintained air contact and the plants continued to grow. As cuticle properties also influence water loss and pathogen infections (Samuels *et al.*, 2008), we also tested the responses of the *drp7* mutant to water deficit and to rice blast disease. The *drp7* mutant showed sensitivity to water deficit (Fig. S10a,b), associated with faster leaf water loss (Fig. S10c) and lower net photosynthesis in air of low humidity (46% RH), compared with Kinmaze (Fig. S10d). The *drp7* mutant was more susceptible than Kinmaze to infection by rice blast (Fig. S11). These results show the wider significance of *LGF1* for stresses beyond its role in leaf gas film retention and tolerance of complete submergence.

Discussion

Hydrophobic leaves with gas film retention are of importance for rice crops. Our study identified *LGF1*/*OsHSD1*, the gene determining this leaf trait. Hydrophobic body surfaces that retain a thin layer of gas when submerged are a mechanism to enhance gas exchange with water, evident both in aquatic invertebrate animals and in some plants (Thorpe & Crisp, 1947; Hebets & Chapman, 2000; Pedersen & Colmer, 2012). However, the molecular genetic mechanism of leaf gas film retention had not been elucidated. As discussed in the following, the *LGF1* allele of *OsHSD1* determines the amount of C30 primary alcohol and therefore the C30 primary alcohol-to-aldehyde ratio, which is of importance for formation of abundant epicuticular wax platelets on leaves of rice, which in turn strongly affects surface hydrophobicity, gas film retention, and underwater photosynthesis during submergence.

Most interestingly, *LGF1*/*OsHSD1* is not a *CER*-like gene, of which seven are known for rice (Fig. S12). The influence of *OsHSD1* on leaf wax composition has been suggested to occur via interactive effects of OsHSD1 on expression of *CER* genes (Zhang *et al.*, 2016). *LGF1*/*OsHSD1* encodes an HSD, being one member of an *HSD* gene subfamily (Fig. S12; Zhang *et al.*, 2016). *LGF1* and *OsHSD1* encode the same gene; however, the two mutant alleles differ for the *drp7* (present study) and the

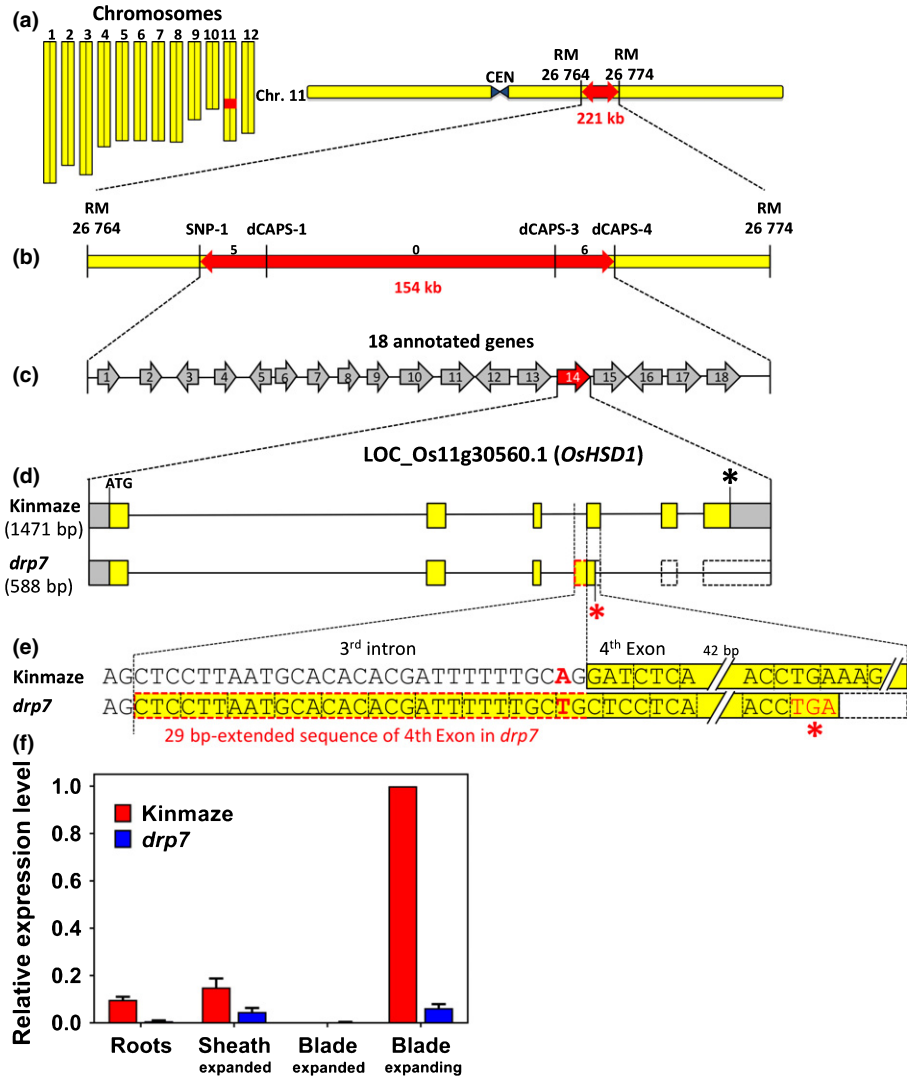

Fig. 3 Identification of *Leaf Gas Film 1* (*LGF1*) which determines leaf gas film retention in rice. (a) The gene associated with gas film loss in the *drp7* mutant was rough-mapped to the long-arm of chromosome 11. (b–d) The physical map of the candidate region on chromosome 11 (numbers above the red arrow indicate number of recombinants) (b) and the candidate region (c) contains 18 annotated genes (TIGR), with the candidate gene identified as *OsHSD1* by genomic DNA sequence comparison of Kinmaze and the *drp7* mutant (d). (e) Gene structure and sequence showing the mutation site at the 3′- splicing site in the third intron of *OsHSD1* in the *drp7* mutant (single nucleotide polymorphism of 'T' in *drp7* vs 'A' in Kinmaze) resulted in a stop codon (indicated with *) as well as a 29 bp upstream extension of the fourth exon. The resulting truncated mRNA presumably explains the loss-of-function mutation (i.e. loss of leaf gas films) and so we named this allele *Leaf Gas Film 1* (*LGF1*). (f) The expression level (transcript abundance) of *LGF1* in roots, youngest fully expanded leaf sheath and leaf blade, and expanding leaf blade of Kinmaze and the *drp7* mutant (see Supporting Information Fig. S5 for diagram showing these organs). Values are means ± SD ($n = 3$), with each replicate being from a different plant. Ubiquitin (LOC_Os04g53620) was used as an internal control for normalization. Expression level in the expanding leaf blade of Kinmaze was used for reference expression level. See Table S1 for data on primers.

oshsd1 (Zhang *et al.*, 2016) mutants. Moreover, the leaf wax composition of the *drp7* mutant showed specific changes in the levels of only two C30 wax components (verified also for the *LGF1* complementation lines in the *drp7* background), whereas the *oshsd1* mutant showed changes in the levels of several other wax components (Zhang *et al.*, 2016). The differences in wax composition between the two mutants could be a result of the substantial differences in the mutation events: the predicted truncated protein in the *drp7* mutant (stop codon in the fourth exon) vs the larger predicted protein as a result of an amino acid deletion and an amino acid substitution in the *oshsd1* mutant. In addition, the expression level of *LGF1* in the *drp7* mutant was substantially lower in various tissues than in Kinmaze (Fig. 3f), indicating that the *drp7* mutant has a putative null allele. Thus, the *drp7* mutant differs markedly from the *oshsd1* mutant, which showed lower expression of *OsHSD1* but had the same enzymatic activity as its wild-type (Zhang *et al.*, 2016). Moreover, although leaf hydrophobicity and wax platelet abundance were studied for the *oshsd1* mutant (Zhang *et al.*, 2016), gas film retention was not evaluated, so the present data on the *drp7* mutant extend our knowledge of the *LGF1/OsHSD1* gene and also provide a comprehensive phenotypic analysis of these leaf surface properties

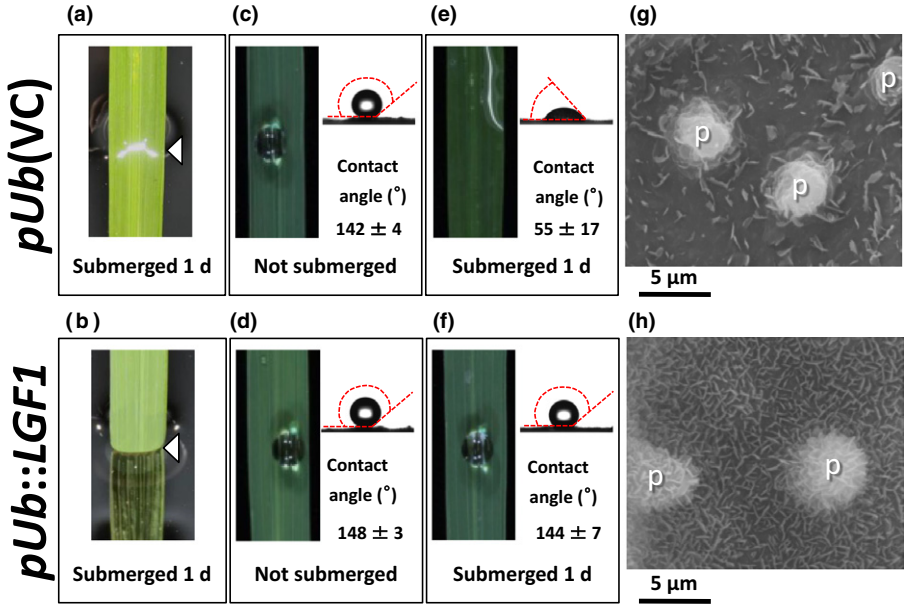

Fig. 4 Complementation test of *LGF1* expressed in the *drp7* mutant background. (a, b) Leaf gas films were not retained on leaves of the vector control lines *pUb*(VC) in the *drp7* background (a), whereas gas film persistence was restored for the *pUb::LGF1* complementation lines (Kinmaze *LGF1* in *drp7* background) as evident after 1 d of submergence (b). When present, gas films are evident as a silvery sheen on the lower half of leaves dipped into water; the white triangle shows the water level. (c, d) The leaf blade surface is initially hydrophobic on the vector control *pUb*(VC) lines and the *pUb::LGF1* complementation lines, as demonstrated by the water droplet test. (e, f) Within 1 d of plant submergence, the leaf surface of the vector control *pUb*(VC) lines had become hydrophilic, but leaves remained hydrophobic for the *pUb::LGF1* complementation lines. (c–f) Contact angles of water droplet and leaf surface were measured, as indicated by the dotted red lines. (g, h) Scanning electron micrographs of leaf blades showed a relatively low density of epicuticular wax platelets for the vector control *pUb*(VC) line, but that epicuticular wax platelet density had increased for the *pUb::LGF1* complementation lines. The numerous, small white 'flecks' on the leaf surface are the epicuticular wax platelets, whereas the larger circular-like structures (top view of a dome-like protrusion) are papillae (labelled 'p'). All measurements were on the adaxial side of leaf 7 of first-generation (T_0) plants (each replicate used an individual T_0 plant from an independent transformation event). Contact angles are of a 1 µl water droplet (side views in the figure; the top views used larger drops of water) and are the angles (relative to the horizontal) at which the water–air interface of the droplet meets the leaf surface. Data in (c)–(f) are means ± SD ($n = 4$) with each replicate being a leaf blade segment from a different plant. The greater mean contact angle of water droplets on the *pUb::LGF1* complementation lines was significant ($P < 0.0001$; *t*-test) when compared with the vector control *pUb*(VC) line.

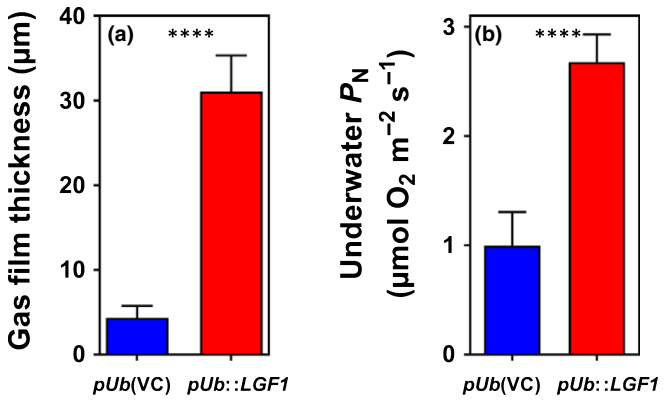

Fig. 5 Complementation test of *LGF1* expressed in the *drp7* mutant background. (a) Leaf gas film persistence was restored for the *pUb::LGF1* complementation lines (Kinmaze *LGF1* in *drp7* background) as demonstrated by measurements of gas film thickness after 1 d of submergence. (b) Restoration of leaf gas films had a positive effect on underwater net photosynthesis (P_N) by the *pUb*::*LGF1* complementation lines as compared with the vector control *pUb*(VC) lines (*drp7* background). Measurements were on the youngest fully expanded leaf of first-generation (T_0) plants (each replicate used an individual T_0 plant from an independent transformation event). Data are means ± SD ($n = 4$ in both (a) and (b)) with each replicate being a leaf segment from a different plant. Significant difference (*t*-test): ****, $P < 0.0001$.

(Fig. 1) as associated with leaf gas film retention and photosynthesis when submerged (Fig. 2).

The finding that *LGF1* specifically controls abundance of C30 primary alcohol (Figs 6a,b, S8) is a new insight into wax biosynthesis in rice. The specific increases in C30 aldehyde abundance and decrease in C30 primary alcohol for the *drp7* mutant, and the recovery back to the wild-type levels in the *LGF1* complementation lines (Figs 6a,b, S8), lead us to propose two hypotheses regarding the role of the LGF1 protein based on current knowledge of wax biosynthesis (Samuels *et al.*, 2008), the relevant components of which are summarized in Fig. 7. The LGF1 protein is hypothesized to be involved in conversion of C30 aldehyde to C30 primary alcohol (Hypothesis 1) or of C30 very long-chain fatty acyl-CoA to C30 primary alcohol (Hypothesis 2). In the case of Hypothesis 1, if there is conversion of C30 aldehyde to the C30 primary alcohol (for this possibility in other organisms, see Kunst & Samuels (2003) and Zhou *et al.* (2014)) then inhibition of this reaction would result in accumulation of the C30 aldehyde and a deficit of the C30 primary alcohol. In the case of Hypothesis 2, if conversion of the C30 very long-chain fatty acyl-CoA directly to the primary alcohol is diminished (for this conversion in other organisms, see Samuels *et al.* (2008) and Wang *et al.* (2015)) then the flux along the alternative

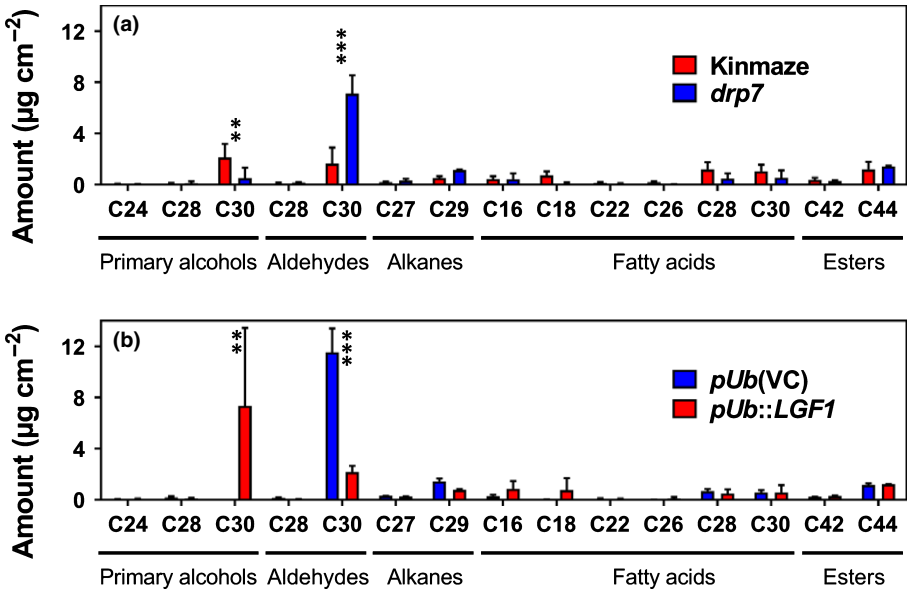

Fig. 6 Leaf wax composition shows an altered balance between C30 aldehyde and C30 primary alcohols in the *drp7* mutant and restoration of composition in the *pUb::LGF1* complementation lines. (a) Cuticular wax composition of leaf blades of Kinmaze and *drp7*. (b) Cuticular wax composition of leaf blades of the *pUb*::*LGF1* complementation lines (Kinmaze *LGF1* in *drp7* background) and the vector control *pUb*(VC) lines (*drp7* background). Measurements were on leaf 13, using GC-flame ionization detection analyses. For the complementation lines and vector control lines, measurements were on leaves of first-generation (T_0) plants (each replicate used an individual T_0 plant from an independent transformation event). Values are means ± SD ($n = 3$ in both (a) and (b)) with each replicate being leaf blade segments from a different plant. Significant differences (multiple *t*-tests): **, $P < 0.01$; ***, $P < 0.001$. Wax composition was also measured for younger plants and again showed an altered balance between C30 aldehyde and C30 primary alcohols in the *drp7* mutant (Fig. S8).

pathway to C30 aldehyde would predominate. Thus, either of these two cases is consistent with the observed changes in the C30 wax components as found here for the *drp7* mutant and the *LGF1* complementation lines (Figs 6a,b, S8).

In addition to the influence of epicuticular wax platelets on leaf hydrophobicity (Marmur, 2003; Koch & Barthlott, 2009), papillae also contribute to leaf hydrophobicity (Koch & Barthlott, 2009), but this feature did not differ between the *drp7* mutant and Kinmaze (Figs 1g,h, S2). Development of papillae on rice leaves is regulated by *OsRopGEF10* (Yoo *et al.*, 2011). Our observation of gas films initially being present on leaves of the *drp7* mutant upon submergence, but disappearing during the first day under water, might be explained by the papillae providing initial leaf hydrophobicity, with subsequent ingress of water onto the leaf surface layer because of the low abundance of epicuticular wax platelets.

The identification of *LGF1*/*OsHSD1*, a gene controlling leaf gas film retention when under water, adds to existing knowledge of the genetic basis of submergence tolerance in rice (e.g. Sub1A; Xu *et al.*, 2006). Leaf gas films contribute to plant submergence tolerance as the resistance is lower for inward diffusion of CO_2 and O_2 into the leaves (Verboven *et al.*, 2014), which aids photosynthesis and internal aeration during complete submergence (Pedersen *et al.*, 2009; Winkel *et al.*, 2013, 2016). The adaxial and abaxial surfaces of rice leaves are similarly hydrophobic and both surfaces possess gas films when submerged (Pedersen *et al.*, 2009); the *drp7* mutant lost gas films from both leaf sides soon after submergence and gas film retention was restored on both sides for leaves of the *LGF1*/*OsHSD1* complementation lines. The CO_2 response curve of underwater net photosynthesis by Kinmaze and the *drp7* mutant at 1 d after submergence showed that at low external concentrations, the rate of CO_2 entry into leaves was 8.2-fold higher for Kinmaze than for the *drp7* mutant (Fig. 2c). This 8.2-fold difference between leaves of Kinmaze (gas films present) and the *drp7* mutant (gas films absent) compares with the three- to fivefold higher CO_2 entry into leaves with gas films present as compared with those for which gas films had been experimentally removed using a dilute detergent (Pedersen *et al.*, 2009; Winkel *et al.*, 2013). Importantly, when under water with 100 mmol CO_2 m^{-3} (a concentration relevant in field situations; Colmer *et al.*, 2011) the leaves of Kinmaze had a 4.5-fold higher rate of net photosynthesis compared with those of the *drp7* mutant (Fig. 2c), and the *LGF1*/*OsHSD1* complementation lines in the *drp7* background had a 2.7-fold higher rate of underwater net photosynthesis compared with those of the vector control lines (Fig. 5b). The present study of the *drp7* mutant and the *LGF1*/*OsHSD1* complementation lines, together with previous work which experimentally manipulated leaf gas films (Pedersen *et al.*, 2009; Winkel *et al.*, 2013) or modelled the function of this leaf trait (Verboven *et al.*, 2014), demonstrates the significance of leaf gas film retention for underwater photosynthesis and submergence tolerance of rice.

In conclusion, *LGF1*/*OsHSD1* controls C30 primary alcohol synthesis, and the resulting C30 primary alcohol-to-aldehyde ratio influences the abundance of epicuticular wax platelets, which determines leaf hydrophobicity and confers retention of gas films on submerged leaves. This trait is essential for survival of rice even in paddy field conditions and contributes to

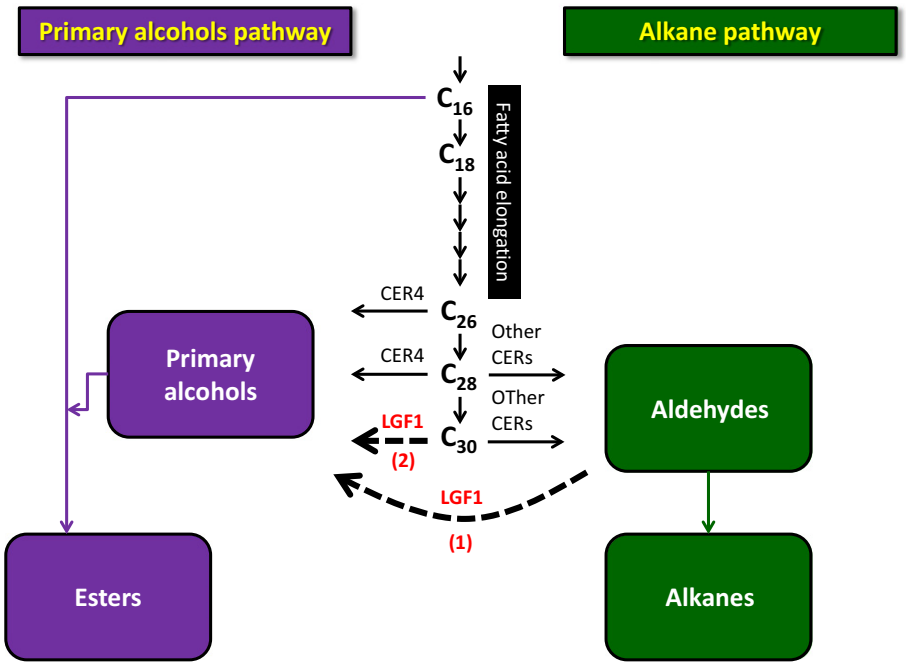

Fig. 7 Simplified summary scheme of wax synthesis pathways in rice (based on the scheme for Arabidopsis presented by Samuels *et al.* (2008) and other information as described in this caption) and with possible involvement of LGF1 (*LGF1*/*OsHSD1*) indicated. Fatty acid elongation produces very long-chain fatty acyl-CoAs and these enter either the primary alcohols pathway (Samuels *et al.*, 2008) or the alkane pathway (Kunst & Samuels, 2003). Primary alcohols and very long-chain fatty acids are components in wax esters (Heilmann *et al.*, 2012). In Arabidopsis, the alkane pathway continues to ketones (Rashotte *et al.*, 2001; Greer *et al.*, 2007), but this is not shown in the diagram as this has not been described for rice. In the primary alcohol pathway, several *fatty acyl reductase* (*FAR*) genes have been identified. These include *ECERIFERUM4* (*CER4*), which encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in cuticular wax production in Arabidopsis to produce C26 and C28 primary alcohols from the respective very long-chain fatty acyl-CoA (same C number), which was identified using a *cer4* mutant that had decreased primary alcohols and increased VLCFAs (Rowland *et al.*, 2006). Paths (1) and (2) within the scheme refer to the hypothesized actions of LGF1 as discussed in the main text.

submergence tolerance. Discovery of *LGF1* provides an opportunity in the future to better understand variation among rice genotypes of leaf gas film retention (e.g. Winkel *et al.*, 2014) and possibly to identify alleles that confer higher *LGF1*/*OsHSD1* expression (and protein abundance) and/or higher activity of the enzyme, which could be related to epicuticular wax platelet abundance and the duration of gas film retention on leaves during submergence. If any allelic variation in *LGF1*/*OsHSD1*, either natural or induced, is associated with leaf gas film retention, this could be a future breeding target in addition to *Sub1* (Ismail *et al.*, 2013) for further improvement of rice submergence tolerance and yield stability of rice crops in flood-prone areas.

Acknowledgements

We thank Dr Toshihiro Kumamaru from the Faculty of Agriculture, Kyushu University, for providing seeds of Kinmaze and the *drp7* mutant. We thank Kobelco Research Institute Inc. for testing micro-water repellence by E-SEM. We thank Dr Yin Wang from the Institute for Advanced Research, Nagoya University, and Dr Takeshi Ohta from the Graduate School of Bioagricultural Science, Nagoya University, for access to a Li-Cor 6400. This research was supported by SATREPS by JICA and JST, JSPS KAKENHI grant number JP16K18565, and a MEXT Grant-in-Aid for Scientific Research on Innovative Areas (17H06473). T.D.C., O.P. and A.I.M. thank Nagoya University for visitor support. Components of this research were supported by grants from the Villum Foundation, the Danish Research Council and the Sassakawa Foundation.

Author contributions

Y.K., K.N., M.S., H.O., J-i.I., A.Y., O.P., T.D.C. and M.A. designed the research; Y.K., M.S., P.D.H., H.Q., K.N., Y.M., Y.T., T.K., N.H., S.A., K.S., Y.S-S., A.I.M., O.P., T.D.C. and M.A. performed the experiments; Y.K., P.D.H., H.Q., K.N., K.S., Y.S-S. and O.P. analysed the data; and Y.K., K.N., A.I.M., O.P., T.D.C. and M.A. wrote the manuscript. All authors offered suggestions on various drafts of the manuscript.

ORCID

Ole Pedersen http://orcid.org/0000-0002-0827-946X
Timothy David Colmer http://orcid.org/0000-0002-3383-9596
Motoyuki Ashikari http://orcid.org/0000-0002-8945-2366

References

Barthlott W, Neinhuis C. 1997. Purity of the sacred lotus, or escape from contamination in biological surfaces. *Planta* **202**: 1–8.

Chen G, Komatsuda T, Ma JF, Nawrath C, Pourkheirandish M, Tagiri A, Hu Y-G, Sameri M, Li X, Zhao X. 2011. An ATP-binding cassette subfamily G full transporter is essential for the retention of leaf water in both wild barley and rice. *Proceedings of the National Academy of Sciences, USA* **108**: 12354–12359.

Colmer TD, Armstrong W, Greenway H, Ismail AM, Kirk GJD, Atwell BJ. 2014. Physiological mechanisms of flooding tolerance in rice: transient complete submergence and prolonged standing water. *Progress in Botany* **75**: 255–307.

Colmer TD, Pedersen O. 2008. Underwater photosynthesis and respiration in leaves of submerged wetland plants: gas films improve CO_2 and O_2 exchange. *New Phytologist* **177**: 918–926.

Colmer TD, Winkel A, Pedersen O. 2011. A perspective on underwater photosynthesis in submerged terrestrial wetland plants. *AoB Plants* **2011**: plr030.

FAO. 2015. *Rice production 1993–2013*. Geneva, Switzerland: Food and Agriculture Organisation of the United Nations.

Greer S, Wen M, Bird D, Wu X, Samuels L, Kunst L, Jetter R. 2007. The cytochrome P450 enzyme CYP96A15 is the midchain alkane hydroxylase responsible for formation of secondary alcohols and ketones in stem cuticular wax of Arabidopsis. *Plant Physiology* **145**: 653–667.

Hebets EA, Chapman RF. 2000. Surviving the flood: plastron respiration in the non-tracheate arthropod *Phrynus marginemaculatus* (Amblypygi: Arachnida). *Journal of Insect Physiology* **46**: 13–19.

Heilmann M, Iven T, Ahmann K, Hornung E, Stymne S, Feussner I. 2012. Production of wax esters in plant seed oils by oleosomal cotargeting of biosynthetic enzymes. *Journal of Lipid Research* **53**: 2153–2161.

Herzog M, Konnerup D, Pedersen O, Winkel A, Colmer TD. 2017. Leaf gas films contribute to rice (*Oryza sativa*) submergence tolerance during saline floods. *Plant, Cell & Environment*. doi: 10.1111/pce.12873

Hiei Y, Ohta S, Komari T, Kumashiro T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant Journal* **6**: 271–282.

Hood EE, Helmer GL, Fraley RT, Chilton MD. 1986. The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. *Journal of Bacteriology* **168**: 1291–1301.

Islam MA, Du H, Ning J, Ye H, Xiong L. 2009. Characterization of *Glossy1*-homologous genes in rice involved in leaf wax accumulation and drought resistance. *Plant Molecular Biology* **70**: 443–456.

Ismail AM, Singh US, Singh S, Dar MH, Mackill DJ. 2013. The contribution of submergence-tolerant (Sub1) rice varieties to food security in flood-prone rainfed lowland areas in Asia. *Field Crops Research* **152**: 83–93.

Jassby AD, Platt T. 1976. Mathematical formulation of the relationship between photosynthesis and light for phytoplankton. *Limnology and Oceanography* **21**: 540–547.

Koch K, Barthlott W. 2009. Superhydrophobic and superhydrophilic plant surfaces: an inspiration for biomimetic materials. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences* **367**: 1487–1509.

Koch K, Bhushan B, Barthlott W. 2009. Multifunctional surface structures of plants: an inspiration for biomimetics. *Progress in Materials Science* **54**: 137–178.

Kunst L, Samuels A. 2003. Biosynthesis and secretion of plant cuticular wax. *Progress in Lipid Research* **42**: 51–80.

Mao B, Cheng Z, Lei C, Xu F, Gao S, Ren Y, Wang J, Zhang X, Wang J, Wu F. 2012. Wax crystal-sparse leaf2, a rice homologue of WAX2/GL1, is involved in synthesis of leaf cuticular wax. *Planta* **235**: 39–52.

Marmur A. 2003. Wetting on hydrophobic rough surfaces: to be heterogeneous or not to be? *Langmuir* **19**: 8343–8348.

Nagai K, Kuroha T, Ayano M, Kurokawa Y, Angeles-Shim RB, Shim J-H, Yasui H, Yoshimura A, Ashikari M. 2012. Two novel QTLs regulate internode elongation in deepwater rice during the early vegetative stage. *Breeding Science* **62**: 178–185.

Neinhuis C, Barthlott W. 1997. Characterization and distribution of water-repelent, self-cleaning plant surfaces. *Annals of Botany* **79**: 667–677.

Park J-J, Jin P, Yoon J, Yang J-I, Jeong HJ, Ranathunge K, Schreiber L, Franke R, Lee I-J, An G. 2010. Mutation in *Wilted Dwarf and Lethal 1* (*WDL1*) causes abnormal cuticle formation and rapid water loss in rice. *Plant Molecular Biology* **74**: 91–103.

Pedersen O, Colmer TD. 2012. Physical gills prevent drowning of many wetland insects, spiders and plants. *Journal of Experimental Biology* **215**: 705–709.

Pedersen O, Colmer TD, Sand-Jensen K. 2013. Underwater photosynthesis of submerged plants – recent advances and methods. *Frontiers in Plant Science* **4**: 140.

Pedersen O, Rich SM, Colmer TD. 2009. Surviving floods: leaf gas films improve O_2 and CO_2 exchange, root aeration, and growth of completely submerged rice. *Plant Journal* **58**: 147–156.

Qin B-X, Tang D, Huang J, Li M, Wu X-R, Lu L-L, Wang K-J, Yu H-X, Chen J-M, Gu M-H. 2011. Rice *OsGL1-1* is involved in leaf cuticular wax and cuticle membrane. *Molecular Plant* **4**: 985–995.

Rashotte AM, Jenks MA, Feldmann KA. 2001. Cuticular waxes on eceriferum mutants of *Arabidopsis thaliana*. *Phytochemistry* **57**: 115–123.

Raskin I. 1983. A method for measuring leaf volume, density, thickness, and internal gas volume. *HortScience* **18**: 698–699.

Raskin I, Kende H. 1983. How does deep water rice solve its aeration problem? *Plant Physiology* **72**: 447–454.

Rowland O, Zheng H, Hepworth SR, Lam P, Jetter R, Kunst L. 2006. *CER4* encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in cuticular wax production in Arabidopsis. *Plant Physiology* **142**: 866–877.

Samuels L, Kunst L, Jetter R. 2008. Sealing plant surfaces: cuticular wax formation by epidermal cells. *Annual Review of Plant Biology* **59**: 683–707.

Satoh H, Iwata N, Omura T. 1983. Gene analysis of some dripping-wet leaf mutants in rice. *Japanese Journal of Breeding* **33**: 242–243.

Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9**: 671–675.

Smart R, Barko J. 1985. Laboratory culture of submersed freshwater macrophytes on natural sediments. *Aquatic Botany* **21**: 251–263.

Thomson D, Henry R. 1995. Single-step protocol for preparation of plant tissue for analysis by PCR. *BioTechniques* **19**: 394–397.

Thorpe WH, Crisp DJ. 1947. Studies on plastron respiration; the respiratory efficiency of the plastron in *Aphelocheirus*. *Journal of Experimental Biology* **24**: 270–303.

Verboven P, Pedersen O, Ho QT, Nicolaï BM, Colmer TD. 2014. The mechanism of improved aeration due to gas films on leaves of submerged rice. *Plant, Cell & Environment* **37**: 2433–2452.

Wang Y, Wang M, Sun Y, Wang Y, Li T, Chai G, Jiang W, Shan L, Li C, Xiao E. 2015. FAR5, a fatty acyl-coenzyme A reductase, is involved in primary alcohol biosynthesis of the leaf blade cuticular wax in wheat (*Triticum aestivum* L.). *Journal of Experimental Botany* **66**: 1165–1178.

Winkel A, Colmer TD, Ismail AM, Pedersen O. 2013. Internal aeration of paddy field rice (*Oryza sativa* L.) during complete submergence – importance of light and floodwater O_2. *New Phytologist* **197**: 1193–1203.

Winkel A, Pedersen O, Ella ES, Ismail AM, Colmer TD. 2014. Gas film retention and underwater photosynthesis during field submergence of four contrasting rice genotypes. *Journal of Experimental Botany* **65**: 3225–3233.

Winkel A, Visser EJW, Colmer TD, Brodersen KP, Voesenek LACJ, Sand-Jensen K, Pedersen O. 2016. Leaf gas films, underwater photosynthesis and plant species distributions in a flood gradient. *Plant, Cell & Environment* **39**: 1537–1548.

Xu K, Xu X, Fukao T, Canlas P, Maghirang-Rodriguez R, Heuer S, Ismail AM, Bailey-Serres J, Ronald PC, Mackill DJ. 2006. *Sub1A* is an ethylene-response-factor-like gene that confers submergence tolerance to rice. *Nature* **442**: 705–708.

Yoo J-H, Park J-H, Cho S-H, Yoo S-C, Li J, Zhang H, Kim K-S, Koh H-J, Paek N-C. 2011. The rice *bright green leaf* (*bgl*) locus encodes OsRopGEF10, which activates the development of small cuticular papillae on leaf surfaces. *Plant Molecular Biology* **77**: 631–641.

Yu D, Ranathunge K, Huang H, Pei Z, Franke R, Schreiber L, He C. 2008. *Wax Crystal-Sparse Leaf1* encodes a β–ketoacyl CoA synthase involved in biosynthesis of cuticular waxes on rice leaf. *Planta* **228**: 675–685.

Zhang Z, Cheng Z-J, Gan L, Zhang H, Wu F-Q, Lin Q-B, Wang J-L, Wang J, Guo X-P, Zhang X *et al.* 2016. *OsHSD1*, a hydroxysteroid dehydrogenase, is

involved in cuticle formation and lipid homeostasis in rice. *Plant Science* **249**: 35–45.

Zhou L, Ni E, Yang J, Zhou H, Liang H, Li J, Jiang D, Wang Z, Liu Z, Zhuang C. 2013. Rice *OsGL1-6* is involved in leaf cuticular wax accumulation and drought resistance. *PLoS ONE* **8**: e65139.

Zhou YJ, Buijs NA, Siewers V, Nielsen J. 2014. Fatty acid-derived biofuels and chemicals production in *Saccharomyces cerevisiae*. *Frontiers in Bioengineering and Biotechnology* **2**: 1–6.

Zhu X, Xiong L. 2013. Putative megaenzyme DWA1 plays essential roles in drought resistance by regulating stress-induced wax deposition in rice. *Proceedings of the National Academy of Sciences, USA* **110**: 17790–17795.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Photographs and conceptual models of rice leaves with or without leaf gas films.

Fig. S2 Leaf blade surface structure of Kinmaze and the *drp7* mutant.

Fig. S3 Net photosynthesis in air, Chl content (SPAD reading) and leaf thickness of Kinmaze and the *drp7* mutant.

Fig. S4 Sequence comparison of *OsHSD1* between Kinmaze and the *drp7* mutant.

Fig. S5 Schematic diagram of a rice plant showing the organs from which RNA was extracted to assess *LGF1* expression (transcript abundances).

Fig. S6 Net photosynthesis in air by the *pUb*::*LGF1* complementation lines (Kinmaze *LGF1* in *drp7* background) and the vector control *pUb*(VC) lines (*drp7* background).

Fig. S7 Main components of leaf waxes (TLC and GC-MS) of Kinmaze and the *drp7* mutant.

Fig. S8 Wax composition (GC-FID) for leaf blades of Kinmaze and the *drp7* mutant.

Fig. S9 Evaluation of Kinmaze and the *drp7* mutant in a flooded paddy field.

Fig. S10 Responses to water deficit of Kinmaze and the *drp7* mutant.

Fig. S11 Susceptibility to rice blast disease for Kinmaze and the *drp7* mutant.

Fig. S12 Phylogenetic analysis of *SDR* (*FAR*) and *HSD* gene families in Arabidopsis and rice.

Table S1 Primers used in this study

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.